Cellular repopulation of human vein allograft bypass grafts

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Purpose: Vein allografts are an alternative conduit for patients lacking available autogenous vein. The ability to develop a neoendothelium is a potential advantage of allografts over other nonautogenous grafts, because endothelial cells have been shown to play numerous essential roles in vessel survival. However, repopulation by endothelial cells has not previously been demonstrated or characterized in human subjects.

Methods: In our prospective trial, 40 patients (20 men, 20 women) underwent cryopreserved saphenous vein bypass grafting procedures for limb salvage. Several patients underwent multiple grafting procedures. All grafts were sampled at implantation. During the 31 month follow-up interval, 22 allografts were explanted at the time of revision or subsequent surgical procedure. All grafts (22 of 22) demonstrated intact endothelium at implantation and explantation. Seventeen explantation biopsy samples (seven from men, 10 from women) from 16 patients (seven men, nine women) were adequate for further histologic and immunofluorescent analysis. Explants were stained with hematoxylin-eosin and immunohistochemical markers to quantitate rejection and also underwent fluorescence in-situ hybridization, with probes for X and Y chromosomes and counterstain for nuclear envelope. Cells were counted as XX, XY, XO, YO, or unstained. The endothelium and vessel walls were analyzed for origin of cells based on sex-mismatched transplants, with sex-matched transplants serving as controls.

Results: Evidence of cellular damage was noted in all explanted allografts, and moderate or severe rejection (lymphocyte infiltrate, +CD3, +CD8, +CR3, cytotoxic granules) was noted in six explanted allografts (29%). All allografts demonstrated intact endothelium (complete or partial), at the time of both implantation and explantation. Sex-matched (male to male) control explants showed only male cells, as expected. Male donor–female recipient transplants showed complete repopulation by recipient (female) cells in nine of 10 cases (90%), whereas one case (10%) demonstrated partial repopulation (a mosaic of male and female cells). One patient's slides were unreadable. Findings in cells of the allograft wall were identical to those of the endothelium (nine recipient-only cells and one mosaic). Complete absence of donor cells was noted as early as 1 week after implantation, but mosaicism was demonstrated in one patient 3 months after grafting. No relationship could be demonstrated between repopulation and time ($P > .05$), quantity of rejection ($P > .05$), or donor age ($P > .05$).

Conclusion: Both the endothelial lining and vessel wall of venous allografts repopulate with cells of recipient origin, resulting in either a completely novel cellular constituency or a mosaic of host and donor cells. The loss of donor cells may be mediated by apoptosis or rejection, and the rate of migration of repopulating host cells is, at this point, unclear. Although the development of a completely endothelial-lined conduit offers a potential advantage over other alternative conduits, the functional status of the neoendothelium and repopulated vessel wall and their role in maintenance of allograft patency require further investigation. (J Vasc Surg 2000;31:994-1002.)

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Competition of interest: nil.

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Table I. Significant correlations ($p < 0.05$) between markers for 22 explanted allografts*

Variable	*Positive Correlation*	*Negative Correlation*
Graft patency duration	—	Factor VIII, CD31
Azathioprine immunosuppression	—	TIA-1
Cellular infiltrate	Capillary ingrowth, LCA, CD3, CD8, CR3	—
Mural hemorrhage	Intimal thickening, TIA-1	—
Medial necrosis	Intimal thickening, TIA-1	—
Capillary ingrowth	Cellular infiltrate, LCA, CD3, CD8, CR3, CD31, factor VIII	—
Intimal thickening	Mural hemorrhage, mural necrosis	TIA-1, factor VIII
LCA	Cellular infiltrate, capillary ingrowth, CD3, CR3, CD31, factor VIII	—
CD3	Cellular infiltrate, capillary ingrowth, LCA, CR3	SMA
CD8	Cellular infiltrate, capillary ingrowth, TIA-1, factor VIII	—
CR3	Capillary ingrowth, LCA, CD3, CD31, factor VIII	—
TIA-1	Mural hemorrhage, mural necrosis, CD8	Intimal thickening, azathioprine immunosuppression
SMA	—	CD3
CD31	Cellular infiltrate, capillary ingrowth, CD56, factor VIII, LCA, CD3	Graft patency duration
Factor VIII	Capillary ingrowth, LCA, CD8, CR3, CD31	Intimal thickening, graft patency duration

*No significant correlations were identified for CD30, CD79, L-26, CD35, or CD56.
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Autogenous vein grafts are universally accepted as the conduit of choice for lower-extremity bypass grafting procedures. However, these veins are often unavailable, because they were used in an earlier bypass grafting procedure or were removed by stripping, creating the need for an alternative. Results with synthetic materials for such procedures have been disappointing.[1] Consequently, allograft and xenograft veins would present attractive substitutes, because they are more readily accessible and offer the advantage of a human endothelium-lined biological conduit.

The experimental use of vein allografts has also been disappointing, demonstrating that they are subject to frequent and early failure, the causes of which may include local injury, hypercoagulability, stasis, and host immune response. In an earlier paper,[2] we demonstrated a strong cell-mediated immune response to venous allografts in human subjects. This response was characterized as predominantly activated T lymphocytes (+CD3, CD8, CR3) containing cytotoxic granules (TIA-1). Presence of these cells correlated with vessel-wall destruction, measured by means of mural hemorrhage and necrosis (Table I). Intimal thickening also occurred in response to injury, and capillary ingrowth furthered the progression of the immune response against the grafts.

T lymphocytes involved in this phenomenon were largely CD8+, suggesting a major histocompatibility complex (MHC) class I-restricted direct immune response, instead of an indirect response through MHC class II and antigen presenting cells. In addition to this class I-restricted response, it has been suggested that endothelial cells may upregulate their production of MHC class II and provide an indirect immune response. In our earlier study, we found that the quantity of endothelial markers (CD31, factor VIII) decreased with time and the rejection response, perhaps because of donor endothelial cell destruction (Table I). Endothelial markers also declined with the duration of graft patency. The loss of endothelium also correlated with an increase in intimal thickening and an increase in activated CD8 T cell infiltrate. It is unclear whether these observations provide evidence of an endothelial role in facilitating or retarding the rejection response.

These correlations suggest that endothelial cells, regardless of their loyalty, may play an important role in the allograft tolerance and rejection processes. It is thus essential that we further our understanding of such interactions. We sought to characterize the origin of the endothelial cells in our explanted grafts, with the hypothesis that posttransplantation repopulation of the endothelium of such allografts may occur. Repopulation of the allograft endothelium by host cells may initially expedite an immune response, or it may suppress this response and thereby decrease transplant failure rates. We

observed repopulation of both endothelial cells and cells of the medial vessel wall in human venous allografts and attempted to correlate such repopulation with time, severity of rejection, and donor age.

MATERIAL AND METHODS

Trial design. In the context of a prospective trial approved by the Human Subjects Committee of the University of Pennsylvania, 20 male and 20 female patients with rest pain and/or tissue loss requiring bypass grafting to pedal or crural outflow and lacking adequate autogenous saphenous vein were randomly assigned to receive (17 patients) or not receive (23 patients) azathioprine (1 mg/kg per day) after cryopreserved vein allograft bypass grafting.

Venous allografts. Cryopreserved saphenous vein allografts were obtained from Cryolife Cardiovascular (Marietta, Ga). Veins were stored in a dimethyl sufoxide solution at –196°C. Single-donor veins only (no composite allografts) were used. All grafts were larger than 4 mm in diameter and were ABO blood group matched to the recipient. Grafts were not HLA-matched, because this technique is not feasible in clinical applications because of limited graft availability. All patients underwent biopsies of the allografts at the time of implantation. Twenty-two patients underwent biopsies of previously implanted allografts at the time of subsequent graft revision or amputation. For patients whose biopsies were performed from thrombosed grafts at the time of subsequent amputation (16 patients), specimens were taken from the juxta-anastomotic region to obtain a portion of graft from the patent "stump." For patients who were undergoing graft revision for repair of a stenosis or excision of a graft aneurysm, patent midgraft specimens were obtained (six patients). Biopsies were fixed in paraffin and stored at 25°C. Further studies were carried out on 17 of these biopsies (in seven men and 10 women); five were unfit for further analysis.

Histologic analysis. Standard hematoxylin and eosin preparations were made of implanted and explanted allografts. Paraffin immunohistochemistry study was performed with the standard avidin-biotin complex technique, capillary gap technology, and a robotic immunostainer (Ventanna-Biotek, Tucson, Ariz). One pathologist (J.E.T.) evaluated hematoxylin and eosin and immunostained slides in a semiquantitative manner. Each explanted graft was tested with antibodies against smooth muscle actin (SMA), CD3, and TIA-1 (DAKO, Carpenteria, Calif). Each graft was also stained with antibodies against factor VIII (DAKO) to quantify the presence of intact endothelium. Scoring was performed according to these criteria: 0, no label and no change; 1+, less than 2% of compartment labeled or affected by feature; 2+, 3% to 10% of compartment labeled or affected by feature; 3+, moderate, 11% to 50% of compartment labeled or affected by feature; 4+, heavy or severe, more than 50% of compartment labeled or affected by feature.

Immunofluorescent analysis. Slides of each allograft biopsy were prepared according to standard protocol, as were slides of human ovary and prostate for use as controls. Directly before use, slides were soaked in 100% xylenes to remove fixative and then washed in 100% ethanol. Slides were then rehydrated with ethanol and pretreated in 30% sodium bisulfate at 72°C for 30 minutes. Tissues were digested in proteinase K (Boehringer Mannheim, 1:3000 in TE buffer), at 37°C for 1 hour. SO CEP X and SG CEP Y (SpectrumOrange Chromosome Enumeration Probe X and Spectrum Green Chromosome Enumeration Probe Y) dual color complementary DNA probes (Vysis, Downers Grove, Ill) were diluted 1 to 10 in CEP hybridization buffer (Vysis). CEP X (alpha satellite) SpectrumOrange hybridizes to the centromere of human chromosome X (bands p11.1 to q11, locus DXZ1), and CEP Y SpectrumGreen to the satellite III sequence of human chromosome Y (band Yq12, locus DYZ1). Probe solutions were denatured in 70% formamide at 73°C for 5 minutes. Slides were removed from proteinase K, and also denatured in 70% formamide at 73°C for 5 minutes. Denatured slides were dehydrated in serial ethanol dilutions and air-dried. Each slide received 10 μL of probe solution. Hybridization took place overnight in a humidified chamber at 42°C. After hybridization, slides were washed in 2x SSC, and then counterstained with 10 μL 4,6-diamidino-2-phenylindole/antifade before microscopy.

Microscopic assessment of allograft repopulation. All stained slides were read with a single trichrome lens (Chroma Technologies DAPI/FITC/Texas Red) by a single investigator (T.R.J.) and analyzed for the presence of specific nuclear staining. One hundred cells (roughly one tenth of the total number of cells per slide) were assessed from each slide from the endothelium and medial vessel wall. Endothelium and media were each analyzed separately. Adventitia was not analyzed because of the injury to that layer, which may have been caused by the ingrowth of donor cells and

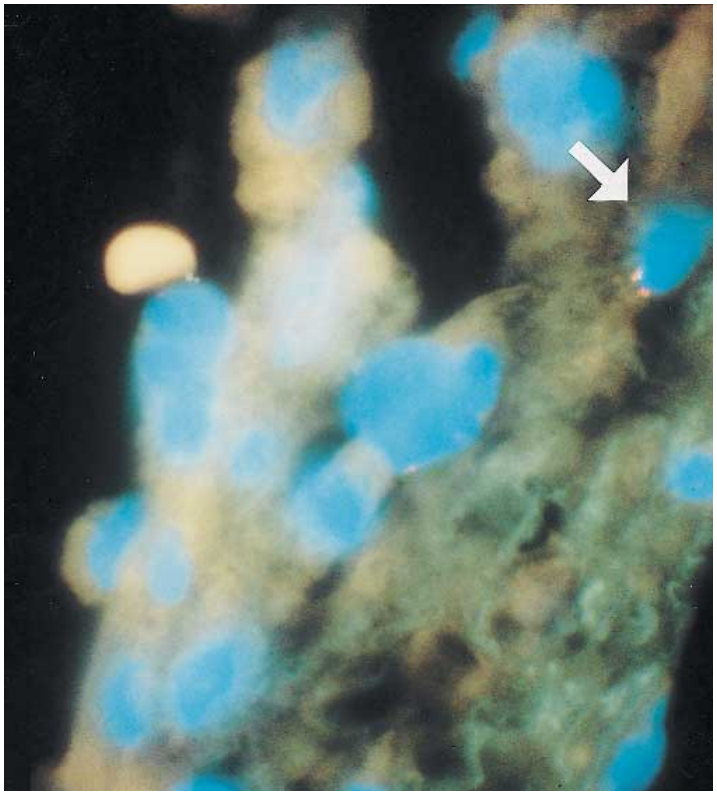

Fig 1. Endothelial repopulation of male donor (XY) saphenous vein allograft by cells from female recipient (XX). The cells are shown at high power (100× with oil) under fluorescence microscopy. The cell at the top of the field (*arrow*) clearly demonstrates two X chromosomes (*red*) within the nucleus (*blue*), denoting female genotype. No cells in the graft stained for the Y chromosome (*green*), whereas 60% stained for XX genotype, 20% stained for single X chromosome only (XO), and 20% did not stain (OO). The three-dimensional orientation of chromosomes in the nucleus caused considerable difficulty in simultaneously photographing both chromosomes, accounting for the apparent lack of staining of all cells in this field.

matrix components, and subsequent injury on explantation. The nucleus of each cell was counted for the number of red or green signals and recorded as either XX, XY, XO, YO, or unstained. The investigator was blinded to both donor and recipient sex status for each slide. Data were compiled and separated between female and male recipients (all allograft donors were men). Data were analyzed by using Statview statistical software (ABACUS Concepts, Berkeley, Calif) to calculate the Pearson correlation coefficient.

RESULTS

Clinical results of the trial were reported previously.[3]

Histologic analysis. All allografts (17 of 17) demonstrated intact endothelium (complete or partial) both at the time of implantation and explantation, as assessed by means of intimal staining with antibodies to factor VIII. All biopsies except one (16 of 17) demonstrated intact smooth muscle cells, as measured by means of anti-SMA antibody staining within the media of vessel walls. All biopsies (17 of 17) demonstrated cells morphologically consistent with smooth muscle cells populating the media.

Evidence of cellular damage was noted in all explanted allografts, and moderate or severe rejection (lymphocyte infiltrate, +CD3, and TIA-1) was noted in six explanted allografts (29%). Further studies characterizing the cellular infiltrate of these grafts have been previously published by the authors[2] and are summarized in the introduction and presented in Table I.

Immunohistochemical analysis. A biopsy of 17 allografts was performed posttransplantation at the time of thrombosis, revision, or subsequent surgical

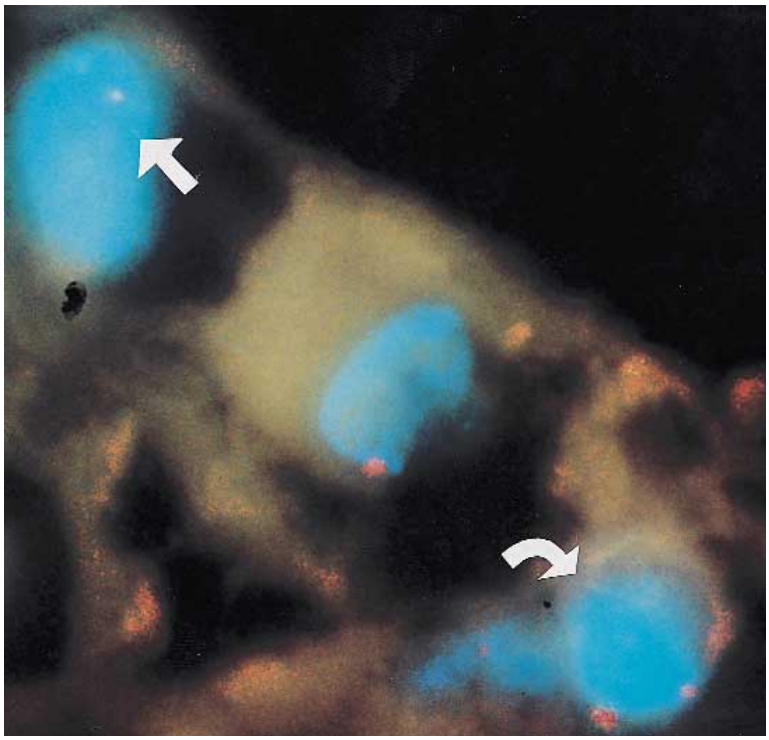

Fig 2. Endothelial cells from a male donor allograft into a female recipient demonstrate mosaicism. The cells are shown at high power (100× with oil) under fluorescence microscopy. The cell at the top of the field (*straight arrow*) stains for X (*red*) and Y (*green*) chromosomes within the nucleus (*blue*), denoting male genotype. The cell at the bottom of the field (*curved arrow*) stains for two X chromosomes, denoting female genotype.

procedure. All allografts were from male donors. A biopsy of seven allografts from male recipients (controls) was performed. There was a biopsy of 10 allografts from female recipients. The endothelia of all male-to-male allografts were populated with more than 80% male cells (XY; small numbers of XX-staining nuclei were present in male-to-male grafts, which most likely represent X staining abnormalities or the loss of Y chromosome because of tissue digestion). Similarly, the vessels walls (media and adventitia) of all male-to-male allografts were populated with more than 80% male cells. In contrast, the endothelia of nine male-to-female allografts were populated by more than 54% female cells (XX; Fig 1). These nine allografts showed no Y chromosome-positive nuclei. The endothelium of one male-to-female allograft was populated with a mosaic of male and female cells, reporting 40% to 50% of cells as male and 30% to 35% of cells as female (20% to 30% of cells noninformative, XO; Fig 2). Similarly, the vessel walls of nine male-to-female allografts were populated almost entirely by female cells (XX; Fig 3). The vessel walls of one male-to-female allograft were populated by a mosaic of 36% to 39% male cells and 22% to 26% female cells, with 22% to 26% of cells noninformative (Fig 4). One female patient's slides were unreadable.

Cells repopulating the media and adventitia were morphologically consistent with smooth muscle cells, and slides from adjacent regions of each biopsy stained consistently for SMA. Most cells demonstrated an elongated "cigar-like" shape. All were mononuclear and did not appear to be inflammatory cells. Further specific immunohistochemical staining to determine histologic cell type could not be performed, because of technological limitations.

The complete absence of donor cells was noted as early as 1 week after implantation, but mosaicism was demonstrated in one patient 3 months after grafting. No relationship could be demonstrated between repopulation and time ($P > .05$), quantity of rejection ($P > .05$), or donor age ($P > .05$). Of the 20 grafts for which data are presented, six grafts were given immunosuppression (four female, two male). No relation was found between the receipt of

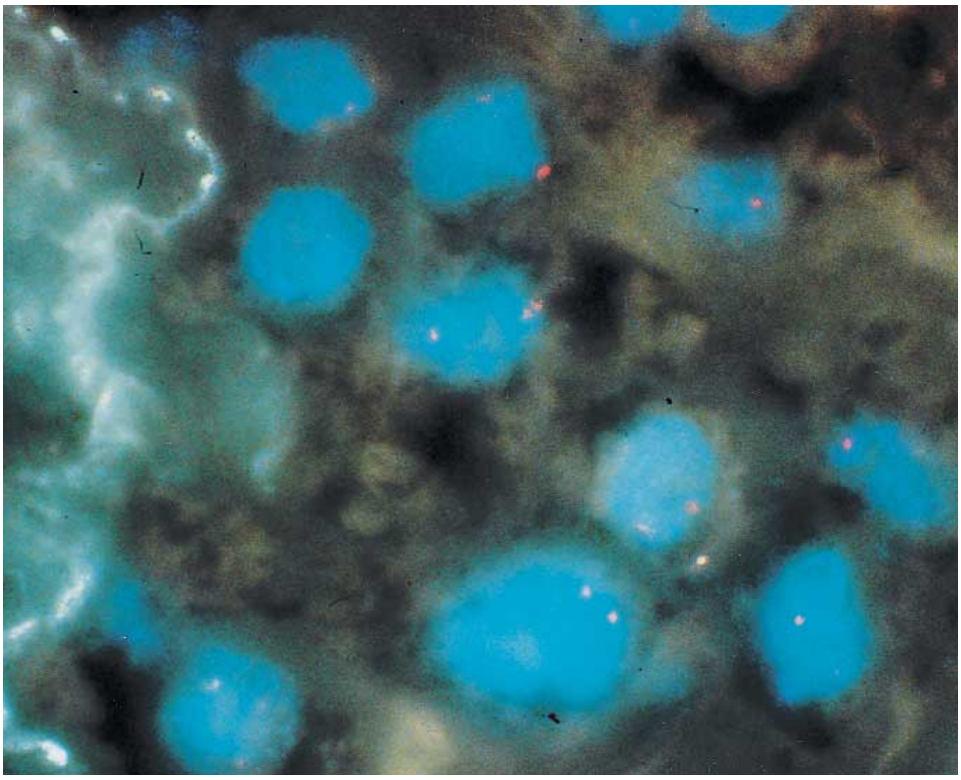

Fig 3. Complete repopulation of the medial compartment of male donor allograft vessel wall by female recipient cells. The cells are shown at high power (100× with oil) under fluorescence microscopy. The cells pictured reside in the media. All cells except one demonstrate staining of two X chromosomes (*red*) within the nucleus (*blue*), suggesting the complete replacement of male cells. No cells in the graft stained for the Y chromosome.

immunosuppression and the degree of cellular repopulation.

DISCUSSION

Since Alexis Carrel successfully performed vascular transplantation in the early part of this century,[4] the search for an ideal vascular graft has continued. Autologous vein has remained the conduit of choice for small vessel procedures.[5] Prosthetic grafts to small vessels perform poorly,[6,7] generating interest in allografts as a biological alternative to prosthetic material. The presence of an endothelial lining in an allograft poses a theoretical advantage over prosthetic alternative grafts; however, venous allografts have demonstrated a low success rate,[8-14] associated with rejection by the host immune system.[2]

Allograft endothelium has been postulated to play a number of important roles in graft survival. An intact endothelium may help to preserve graft integrity, because the expression of prostacyclin inhibits immune cell attachment and diapedesis through the vessel wall. Furthermore, damage to the endothelium allows subsequent exposure of collagen, which is highly immunogenic and a natural site for white blood cell attachment.[15] Endothelium also provides essential functions in intragraft intercellular signaling, mediating dilatation and constriction in response to nitric oxide, hormones, and local blood pressures.

Endothelial cells may also play a role in allograft destruction. These cells may be induced to express MHC class II and can thus function as antigen-presenting cells, expediting rejection by the host immune system. The balance of these functions and their interactions with neighboring cells and the immune system may determine allograft survival.

Initially, venous allografts were hypothesized to be less immunogenic than other allograft tissues.[16] The present study in humans and earlier animal investigations by Perloff et al, Thiede et al, and

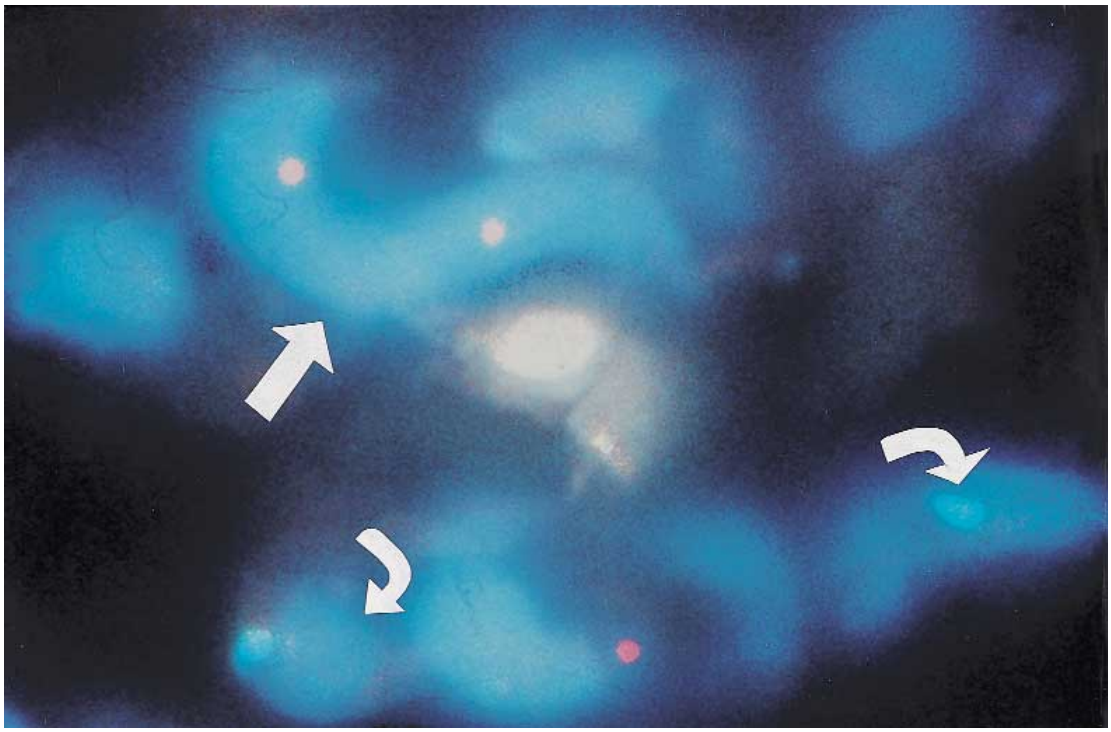

Fig 4. Mosaic repopulation of the medial compartment of male donor allografts. The cells are shown at high power (100× with oil) under fluorescence microscopy. The cells pictured reside in the media. The single S-shaped spindle cell at the top of the picture (*straight arrow*) stains two X chromosomes (female). Two cells at the lower border of the picture (*curved arrows*) stain single Y chromosomes, suggesting male genotype.

Axthelm et al demonstrate that this is not the case.[17-19] We found evidence of cellular damage in all explanted allografts. Venous allografts undergo transplantation rejection much like any other organ. When a graft is placed, an early inflammatory reaction occurs, which causes sloughing of endothelial cells. Such endothelial sloughing exposes the smooth muscle and fibrous tissue of the inner elastic lamina, creating a surface on which host white blood cells and other immune mediators attach and penetrate. This allows host immune recognition of the inner-wall elements and elicits both humoral and cell-mediated immune responses. The strong arm of this response is activated cytotoxic CD8+ T lymphocytes, which can cause direct cellular damage by inducing apoptosis and indirect damage by the release of cytokines that provoke further immune response and inflammation. With time, capillary ingrowth and intimal thickening occur, easing the delivery of mediators and increasing graft injury. The combination of these factors may lead to local injury, thrombogenesis, and, finally, graft occlusion.[2,20]

Cryopreservation has also been suggested to maintain cellular viability and structural integrity of allografts and perhaps to desensitize the immunologic response to transplantation.[21] Cryopreservation and thawing have been shown to result in functional endothelial and smooth muscle cells.[3] Our grafts demonstrated some evidence of injury to the smooth muscle outer layers, but the innermost smooth muscle and endothelium were intact both before implantation and after explantation.

Whether viable cells are even necessary in cryopreserved allografts is questionable, because they may function simply as a matrix on which ingrowth of host cells may occur.[22] Further research will be necessary to determine whether the neoendothelium and repopulated smooth muscle cells are actually functional and whether their function favors patency (anticoagulation) or thrombosis.

This study demonstrates graft damage and a cellular immune infiltrate in grafts concomitant with repopulation of cells in both the endothelial layer and within the vessel wall. Our study used immunofluorescence techniques as a means of demonstrating

infiltration of the endothelium and media by donor endothelial and smooth muscle cells. We postulate that, during an initial rejection response to the donor allotype graft, repopulation by host cells begins to occur, altering the cellular immunotype of the graft itself. This phenomenon is characterized by medial necrosis and cellular infiltrates, as shown in our previous paper[2] and summarized in Table I.

Repopulation by host endothelial and smooth muscle cells and invasion by host immune cells associated with damage and rejection were observed in slides from the same biopsies and thus represent concomitant phenomena. We found complete repopulation of endothelium and smooth muscle by host cells with similar morphology in 80% of our allografts. The route of migration of these repopulating host cells is unclear. It is unlikely that cells simply migrate in from the anastomotic ends of the vessels, because midgraft specimens and specimens from juxta-anastomotic locations demonstrate complete repopulation. Partial repopulation (a mosaic of male and female cells) was observed in one patient. Complete absence of donor cells was noted as early as 1 week after implantation, but mosaicism was demonstrated in one patient 3 months after grafting. No relationship could be demonstrated between repopulation and quantity of rejection ($P > .05$), donor age ($P > .05$), or the use of immunosuppression. The functional significance of mosaicism is not yet determined. Mosaics may represent heterogeneity in the motility of repopulating cells, increased immunologic compatibility between certain individuals, or heterogeneity in the extent of intercellular communication between cells of host and graft.

Presumably repopulation by host cells, which we observed in a number of patients, should decrease graft antigenicity and thereby significantly attenuate the host immune response. Such a scenario has been previously postulated by a number of researchers for liver, kidney, and bone marrow transplants. In the latter setting, donor hematopoietic cells have been shown to reside in a chimeric state with those of host origin. It has been hypothesized that spontaneous microchimerism may be essential for development and maintenance of immunological unresponsiveness to grafts.[23] Confirmation of such a theory could offer insight into the repopulation of venous allografts. Furthermore, similar research could lead to strategies to create an immunologically invisible graft (tolerance), obviating the need for immunosuppression. The functionality of the repopulated graft and its neoendothelium and the exact mechanism of repopulation are unknown and require further investigation.

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